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TITLE

Use of FVIIa or a tissue factor antagonist for regulating cell migration or chemotaxis

FIELD OF INVENTION

A novel cell regulating activity of coagulation factor VII (FVII) or a tissue factor antagonist such as, for example, inactivated coagulation factor VIIa (FVIIai) of cells expressing tissue factor (TF) has been described. The present invention relates to a method for regulating cell migration or chemotaxis by contacting the cell with FVIIa or another TF agonist, or FVIIai or another TF antagonist and determining the migration of said cell. The invention also relates to the use of FVIIa or another TF agonist, or FVIIai or another TF antagonist for the preparation of a medicament for regulation of cell migration in a patient. Moreover the present invention relates to a method of treatment, and a method of detecting the activity of compounds, in particular drug candidates, that interact with cell migration.

BACKGROUND OF THE INVENTION

The extrinsic pathway of blood coagulation is initiated when FVIIa circulating in plasma binds to the integral-membrane protein, tissue factor (TF). The role of TF in blood coagulation has been extensively studied (Camerer, E., A. B. et al. Thromb. Res. 81: 1-41, (1996)). The involvement of FVIIa as a proteolytic enzyme in the blood coagulation cascade is believed to be confined to the extracellular leaflet of TF expressing cells. An intracellular activity of FVIIa was first implied when the sequence of TF showed homology to the cytokine/ interferon- or heamatopoietic receptor superfamily (Bassoon, J. F. Proc. Natl. Acad. Sci. USA 87: 6934-6938, (1990)). The subclass I of the heamotopoietic receptor family includes receptors for growth hormone, prolactin, interleukins 1 to 7, granulocyte- macrophage colony stimulating factors, erythropoitin and thrombopoitin. Subclass II includes TF and receptors for interferon a and b (Wells, J.A., and De Vos, A.M. Annu. Rev. Biomol. Struct. 22: 329-351, (1993)). The resemblance of TF to this class of receptors was further substantiated with the appearance of the crystal structure (Harlos, K., D. M. A. et al. Nature 370: 662-666, (1994), Mueller, Y. A., M. H. et al. Biochemistry 33: 10864-10870 (1994)). Characteristic of this class of cytokine receptors that includes receptors for interferon b and g and IL-10 (Mott, H. R. and Campbell, I. D. Curr. Opin. Struct. Biol. 5: 114-121, (1995)) is that their activation lead to rapid tyrosine phosphorylation of the receptors themselves, as well as a subset of intracellular

proteins. Within minutes after the initial tyrosine phosphorylation an array of mitogen-activated (Ser/Thr) kinases (MAPK) is activated (Whitmarsh, A. J. and Davis, R. J. J. Mol. Med. 74:589-607, (1996)). These kinases are arranged in several parallel signalling pathways (David, M. et al. Science 269, 1721 (1996); Current opin. immunol. 8, 402-11 (1996)). Thorough studies of the putative intracellular signalling capacity of FVIIa have shown that it induce mobilisation of intracellular free calcium (Ca^{2+}) in the human bladder carcinoma cell line, J82, which constitutively express TF and in umbelical vein endothelial cells which were pre-treated with interleukin-1 to express TF (Rottingen, J.-A. et al. J. Biol. Chem. 270: 4650-4660, (1995)), but have failed to show any cytokine-like activation of intracellular tyrosine kinases (Camerer, E., et al. J. Biol. Chem. 271: 29034-29042, (1996)). In conclusion FVIIa is believed, in a TF dependent manner, to induce mobilisation of intracellular Ca^{2+} through activation of phospholipase C (Camerer, E., et al. J. Biol. Chem. 271: 29034-29042, (1996)). The mechanism by which FVIIa activates phospholipase c is not known, but Camerer et al. specifically ruled out tyrosine kinase activation.

SUMMARY OF THE INVENTION

The present invention relates to usage of FVII and/or FVIIa and/or another TF agonist and/or FVIIai and/or another TF antagonist in therapeutic treatment of pathological conditions that can be related to cell migration or treated by specific regulation of cell migration or chemotaxis.

TF has been shown to play a pertinent role in the pathogenesis of a number of diseased states where regulatory interference at the level of chemotaxis is believed to be beneficial.

Thus, diseased states which may be treated are pathological conditions such as atherosclerosis, tumour deposition, tumour growth, tumour invasion, metastasis, or angiogenesis.

LIST OF FIGURES

Fig. 1: Flow cytometric analysis of TF expression in fibroblasts (A). The cells were stained with either a murine monoclonal fluoresceinisothiocyanate (FITC)-conjugated mouse anti IgG-antibody (unfilled area) that was used as negative control or a monoclonal FITC-conjugated anti-tissue factor (TF) antibody (filled area). Fig.1B shows the procoagulant acti-

vity of fibroblasts. Fibroblasts with TF expression generated a 10-fold increase in PCA compared to monocytes without TF expression.

Fig. 2: Effects of FVIIa and FFR-FVIIa on PDGF-BB induced chemotaxis in human fibroblasts. ■ show the chemotactic response of fibroblasts to different concentrations of PDGF-BB. Fibroblasts incubated with 100 nM FVIIa (●) or 100 nM FFR-FVIIa (○) migrated towards different concentrations of PDGF-BB. Results are means and SEM of three separate experiments. P-values less than 0.05,* was considered statistically significant (Student's t test).

Fig. 3 A-D: The influence of different concentrations of FVIIa or FFR-FVIIa on PDGF-BB induced chemotaxis in fibroblasts. ■ show migration of fibroblasts to different concentrations of PDGF-BB. Cells were incubated with 12.5 (A), 25 (B), 50(C) and 100 (D) nM FVIIa (●) or FFR-FVIIa (○) and assayed in the Boyden chamber towards different concentrations of PDGF-BB. Results are mean and SEM of three different experiments. *= *p<0.05*, **= p<0.01 and ***= p<0.001 Student's t test.

Fig. 4: A mixture of three monoclonal antibodies to TF blocks the effects of FVIIa and FFR-FVIIa on PDGF-BB induced chemotaxis in fibroblasts. ■ show migration towards PDGF-BB of fibroblasts without TF antibodies, ● fibroblasts preincubated with TF antibodies and 100 nM FVIIa, and ○ fibroblasts preincubated with TF antibodies and 100 nM FFR-FVIIa. Results are mean and SEM of three separate experiments.

Fig. 5: The influence of FXa on the chemotactic response to PDGF-BB induced by FVIIa. Fibroblasts were preincubated with 200 nM TAP (fig.5 A) (■) or with 0.2-2 μM TAP (fig.5B) (■) and then with 100 nM FVIIa (●). TAP was present during the entire experiments. Chemotaxis was induced by different concentrations of PDGF-BB (A) or by 0.1 ng/ml PDGF-BB (B). Results are mean and SD of two separate experiments.

Fig. 6: The influence of thrombin on the chemotactic response to PDGF-BB induced by FVIIa. Fibroblasts were preincubated with 5 U/mL (final concentration) Hirudin and then with 100 nM FVIIa. Hirudin was present during the entire experiments. Chemotaxis was induced by different concentrations of PDGF-BB. ■ show cells incubated with Hirudin alone and ● cells with Hirudin and FVIIa. Results are mean and SD of two separate experiments.

Fig. 7: Effect of inhibition of PI3'-kinase on chemotaxis in fibroblasts incubated with FVIIa. Cells were preincubated with varying concentrations of LY294002 for 30 min at 37°C, and then with 100 nM FVIIa (●) or without FVIIa (■). The inhibitor was present throughout the chemotaxis assay. Chemotaxis was induced by 0.1 ng/mL PDGF-BB. Results are mean and SD of two separate experiments.

Fig. 8: Effect of inhibition of PLC on chemotaxis in fibroblasts incubated with FVIIa. Cells were incubated with varying concentrations of U73122 (active PLC inhibitor) (A) or U73343 (inactive control) (B) for 30 min at 37°C before incubation with or without 100 nM FVIIa, and then assayed in the Boyden chamber to a concentration gradient of 0.1 ng/mL PDGF-BB. The agents were present during the entire experiments. ■ show cells with U73122 or U73343 alone, ● cells with U73122 or U73343 and FVIIa. Results are mean and SD of two separate experiments.

Fig. 9: Release of inositol trisphosphate (IP_3) from fibroblasts stimulated with FVIIa, FFR-FVIIa alone or in combination with PDGF-BB. Cells were labelled over night with myo [^{3}H] inositol, incubated with or without 100 nM FVIIa or FFR-FVIIa in the absence or presence of 10 ng/mL or 100 ng/mL PDGF-BB. Cells were then analysed for release in IP_3. Open bars show cells without FVIIa or FFR-FVIIa (control), hatched bars show cells with FFR-FVIIa, and black bars show cells incubated with FVIIa.

Fig. 10: Tyrosine phosphorylation of PLC-γ1 in response to PDGF-BB alone (control), FVIIa or FFR-FVIIa in combination with PDGF-BB. Cells were incubated with 100 nM FVIIa or FFR-FVIIa for one hour, and then with or without PDGF-BB at indicated concentrations. Cell were lysed and tyrosine phosphorylation of PLC-γ1 detected as described in methods.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of FVII or FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for inducing or enhancing cell migration in a patient.

In a further aspect the present invention relates to the use of FVII, FVIIa or another TF agonist for the manufacture of a pharmaceutical composition for inducing or enhancing wound healing or angiogenesis.

In a still further aspect the present invention relates to the use of FVIIai or another TF antagonist for the manufacture of a pharmaceutical composition for inhibiting or preventing cell migration in a patient.

In a further aspect the present invention relates to the use of FVIIai or another TF antagonist for the manufacture of a pharmaceutical composition for inhibiting or preventing angiogenesis, metastasis, tumour growth or tumour invasion.

In a further aspect the present invention concerns a method for inducing or enhancing cell migration in a patient, which comprises administering an effective amount of FVII or FVIIa or another TF agonist to said patient.

In a still further aspect the present invention concerns a method for inhibiting or preventing cell migration in a patient, which comprises administering an effective amount of FVIIai or another TF antagonist to said patient.

In a particular embodiment the effective amount is a daily dosage from about 5 μg/kg/day to about 500 μg/kg/day.

In a further embodiment the TF antagonist comprises a modified FVIIa, for example, FFR-FVIIa.

The present invention provides a mechanism for an activity of FVII and/or FVIIa that relates to stimulation of cell migration. Such a mechanism provides the basis for establishing the involvement of FVII and/or FVIIa in pathological conditions in which TF expressing cells like endothelial cells, epithelial cells, fibroblasts, smooth muscle cells and monocytes/ macrophages participate. The invention furthermore provides the basis for identifying specific pharmacological targets that are useful for therapeutic intervention.

Thus, the present invention relates to usage of FVII and/or FVIIa and/or FVIIai in therapeutic treatment of pathological conditions that can be related to cell migration or treated by specific regulation of cell migration.

In another aspect, the present invention relates to a method of detecting drug candidates that regulate cell migration, which method comprise

a) culturing a TF expressing cell;

b) measuring the migration of the cell;

c) incubating the cell with a drug candidate, and

d) measuring the migration of the incubated cell and determining any change in the level of migration compared to the migration measured in step b, such change being indicative of biologically active drug candidate in said cell.

Generally, the blood components which participate in what has been referred to as the coagulation "cascade" are proenzymes or zymogens, enzymatically inactive proteins, which are converted to proteolytic enzymes by the action of an activator, itself an activated clotting factor. Coagulation factors that have undergone such a conversion and generally referred to as "active factors", and are designated by the addition of the letter "a" to the name of the coagulation factor (e.g. factor VIIa).

The term "zinc-chelator" is intended to comprise a compound that binds to factor VIIa and induces replacement of calcium ions with zinc ions within factor VIIa, thereby inhibiting the activity of factor VIIa or tissue factor-factor VIIa complex (TF-FVIIa).

A suitable TF antagonist according to the invention may be a zinc-chelating compound, e.g. a dihydroxamate or a dihydrazide with the hydroxamate or hydrazide groups located relative to each other in such a position that they are able to chelate a zinc ion. The zinc-chelating compound acts in combination with FVIIa. Zn^{2+}-ions exert their inhibitory action in competition with a stimulatory effect of Ca^{2+}-ions. It is predicted that Zn^{2+}-ions displace Ca^{2+}-ions from one or more calcium binding site(s) within FVIIa. Zinc-chelating compounds, e.g. hydroxamates and hydrazides, are capable of acting as powerfull supporters for binding of zinc ions in competition with calcium ions. Specific compounds thereby potentiate zinc inhibition of the activity of the factor VIIa/tissue factor complex. The activity of factor VIIa in complex with tissue factor can be inhibited by a mechanism in which a zinc chelator binds to

factor VIIa and facilitates replacement of Ca^{2+} with Zn^{2+}. By this action the chelator exerts a modulatory effect on TF at the normal concentration of free Ca^{2+} and Zn^{2+} ions in the blood.

Definitions

The term "FVII" means "single chain" coagulation factor VII

The term "Factor VIIa", or "FVIIa" means "two chain" activated coagulation factor VII cleaved by specific cleavage at the Arg152-Ile153 peptide bond. FVIIa, may be purified from blood or produced by recombinant means. It is evident that the practice of the methods described herein is independent of how the purified factor VIIa is derived and, therefore, the present invention is contemplated to cover use of any factor VIIa preparation suitable for use herein. Preferred are human FVIIa.

The term "FVIIai" is intended to mean FVIIa having at least one modification in its catalytic center, which modification substantially inhibits the ability of modified FVIIa to activate FX and FIX. Such modification includes amino acid substitution of one or more of the catalytic triad residues Ser344, Asp142 and His193, and also includes modification of catalytic triad residues with serine protease inhibitors such as organophosphor compounds, sulfanylfluoride, peptide halomethyl ketone or azapeptide. FFR-FVIIa is one example of a FVIIai derivative obtained by blocking of the active centre of FVIIa with the irreversible inhibitor, D-phenylalanine-L-phenylalanine-L-argininine chloromethyl ketone (FFR cmk). Other suitable FVIIai derivates are inactivated FVIIa obtained by blocking the active centre with L-phenylalanine-L-phenylalanine-L-argininine chloromethyl ketone, dansyl-L-phenylalanine-L-phenylalanine-L-argininine chloromethyl ketone, or dansyl-D-phenylalanine-L-phenylalanine-L-argininine chloromethyl ketone, Preferred is FFR-FVIIa.

The term "protein kinase" is intended to indicate an enzyme that is capable of phosphorylating serine and/or threonine and/or tyrosine in peptides and/or proteins.

The term "MAPK signalling pathway" is intended to mean a cascade of intracellular events that mediate activation of Mitogen-Activated-Protein-Kinase (MAPK) and homologues thereof in response to various extracellular stimuli. Three distinct groups of MAP kinases have been identified in mammalian cells: 1) extracellular-regulated kinase (Erk), 2) c-Jun N-

terminal kinase (JNK) and 3) p 38 kinase. The Erk MAP kinase pathway involves phosphorylation of Erk 1 (p 44) and/or Erk 2 (p 42). Activated Erk MAP kinases translocate to the nucleus where they phosphorylate and activate transcription factors including (Elk 1) and signal transducers and activators of transcription (Stat).

The term "FVIIa-induced activation of the MAPK signalling pathway" is intended to indicate that FVIIa binds to TF in a mammalian cell and thereby induce activation of transcription factors Elk1 and Stat elements in a mammalian cell via phosphorylation of MAPK/Erk1/2.

The term "FVIIa mediated intracellular signalling pathway" is intended to indicate a cascade of intracellular events that involve activation of Erk1/2 MAPK.

The term "drug candidate" is intended to indicate any sample which has a biological function or exerts a biological effect in a cellular system. The sample may be a sample of a biological material such as a microbial or plant extract, or it may be a sample containing a compound or mixture of compounds prepared by organic synthesis or genetic techniques.

The term "TF agonist" comprises compounds inducing
a) signal transduction by direct binding to TF (e.g. FVIIa),
b) stimulation of MAPK cascade,
c) abrogation of MAPK inhibition (e.g. PTPase inhibitors),
which agonists are drug candidates as defined above.

The term "TF antagonist" comprises
a) reagents which compete with FVIIa for binding to TF without transmission, e.g. FVIIai,
b) reagents which bind to FVIIa and prevent binding to TF, e.g. Zn hydroxamate,
c) reagents which inhibit signal transduction by interfering with members of the MAPK cascade,
d) reagents which bind to FVIIa/TF and prevent transmission,
e) reagents which bind to FVIIa/TF/FX and prevent transmission,
f) reagents which block human factor X activation catalysed by human tissue factor/factor VIIa complex,
which antagonists are drug candidates as defined above.

The term "pharmacological targets" is intended to indicate a protein that can alter the migration of TF expressing cells.

The term "reporter gene" is intended to indicate a DNA construct that, when transcribed, produces a protein that can be detected.

The term "transcription factor TFC/Elk1" or "transcription factor Elk1" is intended to comprise Elk1 (also known as p62 ternary complex factor, TFC) is an Ets-related transcription factor that mediates growth factor stimulation of the c-fos promoter. Elk1 binds to DNA in part via interaction with Serum Response Factor. Elk1 is a bona fide Erk substrate. SAPKs phosphorylation of Elk1 may mediate transcriptional activation of the fos promotor in response to a variety of stresses.

The term "SRE promoter element" means a DNA sequence that binds transcription factors induced by components present in serum.

The term "TF expressing cell" means any mammalian cell that expresses TF.

The term "protein phosphorylation" is intended to indicate phosphorylation of serine and/or threonine and/or tyrosine in peptides and/or proteins.

Modulation of FVIIa-induced activation of the MAPK signalling pathway in a patient is defined as the capacity of FVIIa or another TF agonist, or FVIIai or another TF antagonist to 1) either increase or decrease ongoing, normal or abnormal, signal transduction, 2) initiate normal signal transduction, and 3) initiate abnormal signal transduction.

In this context, the term "treatment" is meant to include both prevention of an adverse condition and regulation of an already occurring condition with the purpose of inhibiting or minimising the condition. Prophylactic administration of FVIIa or another TF agonist, or FVIIai or another TF antagonist is thus included in the term "treatment".

In this context, the term "one unit" is defined as the amount of factor VII present in 1 ml of normal plasma, corresponding to about 0.5 μg protein. After activation 50 units correspond to about 1 μg protein.

In this context, the term "patient" is defined as any animal, in particular mammals, such as humans, suffering from a condition which may be treated by inhibition or activation of the MAPK signalling pathway.

Abbreviations

TF	tissue factor
FVII	factor VII in its single-chain, unactivated form
FVIIa	factor VII in its activated form
rFVIIa	recombinant factor VII in its activated form
FVIIai	modified (inactivated) factor VII
FFR-FVIIai	factor VII inactivated by reaction with D-Phe-L-Phe-L-Arg chloromethyl ketone

Tissue factor (TF) is the cellular receptor for factor FVIIa (FVIIa) and the complex is principal initiator of blood coagulation. We have studied the effects of FVIIa binding to TF on cell migration and signal transduction of human fibroblasts which express high amounts of TF. Fibroblasts incubated with FVIIa migrated towards a concentration gradient of PDGF-BB at about one hundred times lower concentration than do fibroblasts not ligated with FVIIa. Anti-TF antibodies inhibited the increase in chemotaxis induced by FVIIa/TF. Moreover, a pronounced suppression of chemotaxis induced by PDGF-BB was observed with active site-inhibited FVIIa (FFR-FVIIa). The possibility that hyperchemotaxis was induced by a putative generation of FXa and thrombin activity was excluded.
FVIIa induced the production of inositol-1,4,5-trisphosphate to the same extent as PDGF-BB; the effects of FVIIa and PDGF-BB were additive. FFR-FVIIa did not induce any release of inositol-1,4,5,-trisphosphate. The cellular migration response to PDGF-BB and FVIIa was totally blocked by a PLC-inhibitor, suggesting that activation of PLC is important for the response. Thus, binding of FVIIa to TF can independent of coagulation, modulate cellular responses, such as chemotaxis, and the catalytic activity of FVIIa is necessary.

Tissue factor (TF) is a transmembrane glycoprotein with sequence homology to the class II cytokine/hematopoetic growth factor receptor family which includes receptors for interferon-α, -β and -γ and interleukin-10[1,2]. TF can mediate the activation of the coagulation factor VII

to the activated form FVIIa[3]. The FVIIa/TF complex is the principal initiator of the blood coagulation cascade through activation of both factor IX and factor X[3,4].

Aberrant expression also suggest a central role for TF in thrombosis and inflammation in septicemia, cancer, metastasis and atherosclerosis (reviewed in 5,6). Recently, a role of TF as a true receptor involved in signal transduction was identified. Rottinger et al. showed that 200 nM FVIIa induced Ca^{2+} oscillations in about 30% of human umbilical vein endothelial cells pretreated with IL-1-β to express TF, and in almost 100% of MDCK cells with high constitutive expression of TF[7]. In a subsequent study, FVIIa, and also FXa, induced oscillations in the concentration of intracellular free calcium[8]. Moreover, FVIIa/TF was shown to induce phosphorylation of p44/42 mitogen-activated protein kinase (MAPK) in cells transfected with TF and the active site of FVIIa was found to be mandatory for the response[9]. Other studies have provided evidence that TF functions in tumor cell metastasis, by yet not well-defined mechanism[10]. However, Ott et al. very recently identified actin-binding protein 280 (ABP-280) as a ligand for the TF cytoplasmic domain, providing a molecular pathway by which TF may support tumor cell metastasis[11]. The molecular signals and the biological functions transduced by FVIIa/TF are, however, still poorly understood.

Human fibroblasts have a constitutive expression of TF[1]. These cells also express receptors for platelet-derived growth factor (PDGF)[12]. PDGF induces in its target cells mitogenicity, actin reorganization and directed cell migration (chemotaxis) (for review see 13). We have previously shown that PDGF-BB is an efficient chemotactic factor for human fibroblasts and that the chemotactic response is mediated by the β-receptor class[14]. Therefore, these cells were chosen to study putative signal transduction and cell migration induced by binding of FVIIa to TF.

Below we show for the first time a clear connection between signalling induced by FVIIa binding to TF and the cellular response to a growth factor. We present data that in human fibroblasts the FVIIa/TF complex leads to a hyperchemotactic response to PDGF-BB. Furthermore, active site-inhibited FVIIa (FFR-FVIIa) in a dose-dependent way suppressed the directed migration towards PDGF-BB. By the use of specific inhibitors to PLC and phosphatidylinositol 3'-kinase (PI3'-kinase) we also demonstrate that the hyperchemotactic response towards PDGF-BB induced by FVIIa/TF signalling is dependent upon phospholipase C (PLC) activity but independent of PI3'-kinase. FVIIa and PDGF-BB induced the production of inositol-1,4,5-trisphosphate (IP_3), one of the second messengers released after activation of PLC, in an additiv⁻ manner.

TF is constitutively expressed on the plasma membrane of many extravascular cells, such as stromal fibroblasts in vascular adventitia and in fibrous capsules of liver, spleen and kidney[1]. Thus, expression of TF is found at sites physically separated from the circulating blood and providing a hemostatic envelope. Upon injury this barrier is thought to protect the organism against bleeding. TF can, however, be induced in monocytes/macrophages, vascular smooth muscle cells, endothelial cells and in a number of tumor cells by a variety of agents, including cytokines and growth factors'. Induction at the transcriptional level occurs rapidly after stimulation, identifying TF as a growth-related immediate early gene[17]. Data from studies in which the TF gene was inactivated in mice demonstrated that deficiency of TF results in embryonic lethality due to defective development of blood vessels[18-20]. Insufficient accumulation and differentiation of periendothelial mesenchymal cells to pericytes/primitive smooth muscle cells occurred in the TF deficient embryos[20]. A role of TF in tumor angiogenesis has also been proposed[21]. Thus, accumulating data clearly suggest that TF is involved not only in coagulation but also in several cellular responses, like cell migration.

In this study we have investigated the role of TF as a signalling receptor. We show that human fibroblasts with a constitutive expression of TF upon ligand binding of FVIIa migrate towards extremely low concentrations of PDGF-BB. TF/FVIIa alone did not induce enhanced spontaneous migration, i.e. random migration. Thus, a combination of intracellular signal transduction by FVIIa/TF and the growth factor PDGF-BB was necessary to achieve the motility response. Not only binding to TF, but also the catalytic activity of TF/FVIIa was mandatory, since active-site inhibited FVIIa did not elicit enhanced migration response. Furthermore, inhibitory monoclonal antibodies prevented enhancement of the chemotactic response by FVIIa. We also excluded that indirect signalling occured due to FXa or thrombin, since TAP and Hirudin had no effect on FVIIa/TF induced chemotaxis. We instead found that increasing concentrations of FFR-FVIIa actively inhibited PDGF-BB induced chemotaxis. Fibroblasts incubated with FFR-FVIIa showed completely normal random migration. The inhibitory effect of FFR-FVIIa on PDGF-BB-induced chemotaxis was not observed in the presence of the combination of anti-TF antibodies thereby ruling out the possibility of FFR-FVIIa being toxic. The results suggest rather, that in cells expressing PDGF β-receptors and TF, the FVIIa/TF complex is of importance for the chemotactic response to PDGF-BB. Our observations on the effect of FFR-FVIIa on migration may have a bearing on the findings recently reported by Mueller and Ruf with respect to cancer cell metastasis[22]; they found that increasing doses

FFR-FVIIa to TF expressing cells resulted in a nearly complete inhibition of metastasis in SCID mice.

Our finding that FVIIa increases IP_3 production, and the previously reported data on FVIIa/TF induced Ca^{2+} oscillations especially in MDCK cells, strongly support the notion that PLC is activated by FVIIa/TF signalling in a number of cells. In addition, the hyperchemotactic response in human fibroblasts to PDGF-BB induced by FVIIa/TF was blocked in a dose-dependent way by a PLC-inhibitor. We have previously found a similar hyperchemotactic response to PDGF-BB in PDGF β-receptor Y934F mutant cells which showed increased phosphorylation and activation of PLC-γ1[16]. In these cells, the enhanced phosphorylation of PLC-γ1 correlated with a threefold higher IP_3 production compared to wild-type PDGF β-expressing cells[23]. The combination of FVIIa/TF and PDGF-BB induced about twofold increase in IP_3 production in human fibroblasts. FVIIa/TF-induced IP_3 production, however, did not correlate with phosphorylation of PLC-γ1. Tyrosine phosphorylation of PLC-γ2 induced by FVIIa/TF can not be excluded, but seems unlikely since the expression of PLC-γ2 is very low in human fibroblasts. Moreover, the intracellular part of TF is not endowed with intrinsic protein tyrosine kinase activity. These results suggest that FVIIa/TF induces activation of β and/or δ PLC isozymes. We can not fully exclude that the PLC activation is mediated by FXa or by thrombin. In the assay for IP_3 release the cell culture medium was supplemented with 0.1% FBS containing only about 0.1 nM FXa. We found that a concentration of more than 20 nM FXa is necessary to induce IP_3 production (data not shown). The mechanism by which β or δ PLC isozymes are activated remains to be elucidated. It may be hypothesised that activation involves the cooperation between TF and a membrane associated protein.

Lately, the connection of TF with the cytoskeleton was identified[11,24]. A molecular interaction between the cytoplasmatic domain of TF and the actin filament-binding protein ABP 280 was shown[11]. Furthermore, TF was found to be in close contact with actin and actin filament-binding proteins, such as α-actinin and ABP280 in lamellipodia and ruffled membrane areas in spreading epithelial cells[24]. ABP 280, a member of the filamin subfamily, is required for normal function of lamellipodia and thus highly important for cell motility[25]. PI3'-kinase and PLC isozymes are implicated in chemotactic responses, such as mobilisation of actin-binding proteins[15,25-27]. In previous studies we observed that the PI3'-kinase pathway in PDGF-β receptor induced chemotaxis seems less important in cells with over-expression and enhanced activity of PLC-γ1[16,23]. This was also the case for cells with FVIIa bonded to TF. This indica-

tes that the magnitude of activation of PI3'-kinase and PLC isozymes will determine which of these pathways will dominate. Taken together, our data strongly support the idea that cell migration is one important morphogenic function induced by FVIIa/TF signalling. A cellular migration response is probably mediated in co-operation with different chemotactic factors.

Chemotaxis plays a pivotal role in wound healing, angiogenesis and metastasis. Chemotaxis is also an important component in the development of atherosclerotic plaques. In these processes a variety of cells express TF as well as PDGF and PDGF receptors. Restenosis is a major complication following interventional procedure of obstructed arteries. PDGF has been implicated in the vessel wall's response (neointima formation) to mechanical injury by mediating the migration and proliferation of smooth muscle cells and fibroblasts. We have shown now for the first time that FVIIa binding to TF-expressing cells have an increased chemotactic response to PDGF which is independent of the coagulation.

This finding can explain the efficacy of blocking FVIIa/TF activity in reducing neointima formation in animal models of restenosis[28]. In addition to limit thrombin generation and thrombus formation, and the cellular events caused by thrombin signalling inhibition of FVIIa/TF would also locally inhibit the migration of TF-expressing cells at the site of injury. Our observations suggest that inhibition of FVIIa may become clinically important for regulating these events.

Pharmaceutical administration

The regimen for any patient to be treated with FVIIa or another TF agonist or FVIIai or another TF antagonist as mentioned herein should be determined by those skilled in the art. The daily dose to be administered in therapy can be determined by a physician and will depend on the particular compound employed, on the route of administration and on the weight and the condition of the patient. An effective amount is suitably a daily dosage from about 5 μg/kg/day to about 500 μg/kg/day, preferably from about 10 μg/kg/day to 300 μg/kg/day, more preferred from about 15 μg/kg/day to 200 μg/kg/day, most preferred from about 20 μg/kg/day to 100 μg/kg/day.

The FVIIa or another TF agonist or FVIIai or another TF antagonist should be administered in one single dose, but it can also be given in multiple doses preferably with intervals of 4-6-12 hours depending on the dose given and the condition of the patient.

The FVIIa or another TF agonist or FVIIai or another TF antagonist may be administered intravenously or it may be administered by continuous or pulsatile infusion. FVIIa or another TF agonist or FVIIai or another TF antagonist is preferably administered by intraveneous injections and in an amount of about 100-100,000 units per kg body weight, and preferably in an amount of about 250 - 25,000 units per kg body weight corresponding to about 5-500 μg/kg, a dose that may have to be repeated 2-4 times per 24 hours.

Pharmaceutical compositions

Conventional techniques for preparing pharmaceutical compositions which can be used according to the present invention are, for example, described in Remington's Pharmaceutical Sciences, 1985.

The compositions used according to this invention are prepared by methods known per se by the skilled art worker.

In short, pharmaceutical preparations suitable for use according to the present invention is made by mixing FVII, FVIIa or another TF agonist or FVIIai or another TF antagonist, preferably in purified form, with suitable adjuvants and a suitable carrier or diluent. Suitable physiological acceptable carriers or diluents include sterile water and saline. Suitable adjuvants, in this regard, include calcium, proteins (e.g. albumins), or other inert peptides (e.g. glycylglycine) or amino acids (e.g. glycine, or histidine) to stabilise the purified factor VIIa. Other physiological acceptable adjuvants are non-reducing sugars, polyalcohols (e.g. sorbitol, mannitol or glycerol), polysaccharides such as low molecular weight dextrins, detergents (e.g. polysorbate) and antioxidants (e.g. bisulfite and ascorbate). The adjuvants are generally present in a concentration of from 0.001 to 4% w/v. The pharmaceutical preparation may also contain protease inhibitors, e.g. apronitin, and preserving agents.

The preparations may be sterilised by, for example, filtration through a bacteria-retaining filter, by incorporating sterilising agents into the compositions, by irradiating the compositions, or by heating the compositions. They can also be manufactured in the form of sterile solid compositions which can be dissolved in sterile water, or some other sterile medium suitable for injection prior to or immediately before use.

The present invention is further illustrated by the following examples which, however, are not to be construed as limiting the scope of protection. The features disclosed in the foregoing description and in the following examples may, both separately and in any combination thereof, be material for realising the invention in diverse forms thereof.

EXAMPLES

Example 1

Preparation of FVII

Human purified factor VIIa suitable for use in the present invention is preferably made by DNA recombinant technology, e.g. as described by Hagen et al., Proc.Natl.Acad.Sci. USA 83: 2412-2416, 1986 or as described in European Patent No. 200.421 (ZymoGenetics). Factor VIIa produced by recombinant technology may be authentic factor VIIa or a more or less modified factor VIIa provided that such factor VIIa has substantially the same biological activity for blood coagulation as authentic factor VIIa. Such modified factor VIIa may be produced by modifying the nucleic acid sequence encoding factor VII either by altering the amino acid codons or by removal of some of the amino acid codons in the nucleic acid encoding the natural FVII by known means, e.g. by site-specific mutagenesis.

Factor VII may also be produced by the methods described by Broze and Majerus, J.Biol.Chem. 255 (4): 1242-1247, 1980 and Hedner and Kisiel, J.Clin.Invest. 71: 1836-1841, 1983. These methods yield factor VII without detectable amounts of other blood coagulation factors. An even further purified factor VII preparation may be obtained by including an additional gel filtration as the final purification step. Factor VII is then converted into activated FVIIa by known means, e.g. by several different plasma proteins, such as factor XIIa, IX a or Xa. Alternatively, as described by Bjoern et al. (Research Disclosure, 269 September 1986, pp. 564-565), factor VII may be activated by passing it through an ion-exchange chromatography column, such as Mono Q® (Pharmacia fine Chemicals) or the like.

Example 2

Preparation of FVIIai

Modified factor VII suitable for use in the present invention is made, e.g. as described in International Publications Nos. 92/15686, 94/27631, 96/12800 and 97/47651 ZymoGenetics/Novo Nordisk).

Example 3

Effects of FVIIa and FFR-FVIIa on the chemotactic response of fibroblasts to PDGF-BB

Fibroblasts expressing active TF (Fig. 1) were incubated with 100 nM of FVIIa and seeded in the upper part of the modified Boyden chamber; while media containing 10% FBS and PDGF-BB at different concentrations were added below the 150 μm micropore filter. The migration of the cells under conditions where medium containing 10% FBS without PDGF-BB was added below the filter was used as a measure of random migration, and calculated as 100% migration. A significant migration response was recorded at a concentration of 0.01 ng/ml PDGF-BB in cells stimulated by FVIIa compared to 1 ng/ml PDGF-BB for cells not ligated with FVIIa, i.e. a 100-fold difference in concentration (Fig.2). At 0.01-0.1 ng/ml PDGF-BB the migration response to FVIIa increased dose dependently, starting at 25 nM and with a maximal effect at 50-100 nM FVIIa (Fig.3A-D). No enhancement of random migration was observed after activation with FVIIa. To test whether the proteolytically active FVIIa was mandatory for the hyperchemotactic response to PDGF-BB, fibroblasts were also incubated with 100 nM FFR-FVIIa and assayed in the Boyden chamber in the same way (Fig.2). No increased chemotaxis was observed with FFR-FVIIa at low concentrations of PDGF-BB, 0.01-1 ng/ml. In contrast, a pronounced suppression of chemotaxis induced by 10-50 ng/ml PDGF-BB was achieved by 100 nM FFR-FVIIa (Fig. 2 and 3A-D).

When fibroblasts were preincubated with a mixture of three different TF antibodies and then with FVIIa or FFR-FVIIa, the migration response to PDGF-BB was identical to the response of fibroblasts without the presence of ligand bonded to TF (Fig.4). An irrelevant monoclonal IgG antibody did neither prevent hyperchemotaxis induced by FVIIa nor the inhibition of the migration response induced by FFR-FVIIa (data not shown). The presence of the IgG antibodies or the three TF antibodies did not change random migration of the fibroblasts (data not shown).

Example 4

The hyperchemotactic response is not mediated by FXa or by thrombin

Since FVIIa-induced signal transduction leading to the hyperchemotactic response to PDGF-BB was dependent on the catalytic activity of FVIIa it was important to determine whether signalling occurred directly or via FXa or thrombin generated by the FVIIa/TF complex. The enhanced migration response transduced by FVIIa/TF was not blocked by 0.2-10 μM Tick anticoagulant peptide (TAP), which specifically blocks the active site of FXa and prevents a further activation of the coagulation cascade leading to thrombin formation (Fig. 5A,B). Neither addition of 5 U/ml Hirudin, a specific thrombin inhibitor, had any effect on FVIIa/TF induced hyperchemotaxis (Fig.6). TAP and Hirudin did not influence the migration of fibroblast in response to PDGF without the presence of the ligand FVIIa (Fig.5, 6). Thus, it is unlikely that the effect of FVIIa on chemotaxis is mediated via the activation of FX or thrombin.

Example 5

The hyperchemotactic response to PDGF-BB is influenced by PLC-dependent pathways, but independent of PI3'-kinase.

Activation of PI3'- kinase has recently been shown to be important for PDGF β-receptor induced chemotaxis[15,16]. Therefore, we investigated whether LY294002, a specific PI3'-kinase inhibitor, was able to block the chemotactic response induced by FVIIa/TF signalling. Fibroblasts were pretreated with LY294002 at indicated concentrations for 30 minutes at 37°C before the addition of 100 nM FVIIa and assayed in the Boyden chamber as described. The concentration of PDGF-BB was kept constant at 0.1 ng/ml throughout the assay, i.e. a very low concentration at which FVIIa/TF induced a significant chemotactic response. LY294002 was present during the entire experiments. Fig.7 shows that the migration response to PDGF-BB mediated by FVIIa/TF-signalling was unaffected by the inhibition of PI3'-kinase.

To investigate whether the FVIIa/TF-induced chemotactic response involved the activation of phosphatidylinositol specific phospholipase C (PLC), we preincubated the fibroblasts with different concentrations of U73122, a specific PLC-inhibitor, for 30 minutes at 37°C before adding 100 nM FVIIa; the cells were then subjected to the chemotaxis assay in the presence of the inhibitor. A close analogue, U73343, without effects on PLC was used as negative control. The concentration of PDGF-BB was kept constant at 0.1 ng/ml also in these experiments. Pretreatment of the cells with the active PLC-inhibitor U73122 inhibited the hyperchemotacic response to 0.1 ng/ml PDGF-BB in a dose-dependent way, with a total inhibition at 1 μM (Fig.8). No effect on chemotaxis was observed when the inactive analogue U73343 was used.

Example 6

FVIIa/TF induce activation of PLC

To further explore the importance of PLC activity for the hyperchemotactic response, we also analysed the direct effects of FVIIa/TF on PLC activity in fibroblasts. Activation of PLC leads to production of two second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol. Fibroblasts were incubated with *myo* [^{3}H] inositol overnight, and then with 100 nM FVIIa or FFR-FVIIa for 60 minutes, followed by incubation with or without PDGF-BB at indicated concentrations. Treatment with 100 nM FVIIa alone for 60 minutes induced IP_3 release in fibroblasts at the same level as 10 ng/ml and 100 ng/ml PDGF-BB alone did (Fig.9). Moreover, the combination of 100 nM FVIIa and 10 ng/ml or 100 ng/ml PDGF-BB doubled the IP_3 release. The active site-inhibited FVIIa did not induce release of IP_3. These results clearly show that PLC is activated upon binding of FVIIa to TF.

Example 7

Phosphorylation of PLC-γ1 is not enhanced by TF/FVIIa signalling in fibroblasts

In order to determine whether the PLC-γ1 isoform, which is activated by certain tyrosine kinase receptors, was responsible for the increased PLC activity induced by FVIIa/TF, tyrosine phosphorylation of PLC-γ1 was studied. Fibroblasts were incubated in the absence or presence of 100 nM FVIIa or FFR-FVIIa for one hour, followed by the stimulation with 0, 2, 10 or 100 ng/ml PDGF-BB. After 5 minutes of incubation, the cells were lysed and PLC-γ1 was immunoprecipitated, separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibodies. Whereas a significant increase in tyrosine phosphorylation of PLC-γ1 was recorded with increasing concentrations of PDGF-BB, addition of FVIIa alone to the fibroblasts did not induce any tyrosine phosphorylation of PLC-γ1 (Fig.10). Moreover, the combination of FVIIa and PDGF-BB at different concentrations did not induce any further phosphorylation compared to stimulation with PDGF-BB alone (Fig.10). FFR-FVIIa had no effect on PLC-γ1 tyrosine phosphorylation (Fig.10). Thus, other PLC isoforms than PLC-γ1 are responsible for the increased PLC activity after FVIIa stimulation.

Example 8

Methods

Cell cultures. Human foreskin fibroblasts, AG1518 and AG1523 were grown to confluence in Eagle's MEM supplemented with 10% fetal bovine serum (FBS). Before use, the cells we-

re detached by trypsinization (2.5 mg/ml for 10 min at 37°C), washed in Hank's balanced salt solution, and resuspended in Eagle's MEM with 10% FBS or in Ham's medium supplemented with 0.1% FBS.

Proteins. Human FVIIa (Novo Nordisk A/S, Gentofte, Denmark), was expressed and purified as described[29]. FFR-FVIIa (Novo Nordisk) was obtained by blocking of FVIIa in the active site with D-Phe-L-Phe-L-Arg chloromethyl ketone[30]. Recombinant Tick anticoagulant peptide (TAP) was kindly provided by Dr. P. Vlasuk, Corvas (San Diego, CA). Hirudin was purchased from Sigma. LY294002, U73122 and U73343 were obtained from Biomol (Plymouth Meeting, PA). Anti-TF monoclonal antibodies, TF8-5G9, TF9-5B7 and MTFH-1[31], was a kind gift of Dr. James H. Morrissey, Oklahoma Medical Research Foundation. The phosphotyrosine antibody, PY99 was from Santa Cruz, California.

Flow cytometry. The surface expression of TF was analysed by immunofluorescence with a flow cytometer (Coulter Epics XL-MCL, Beckman Coulter, Fullerton, CA, Coulter Electronics, USA). The instrument was calibrated daily with Immuno-Check™ or Flow Check™ calibration beads (Coulter). For indirect immunofluorescence experiments AG1518 or AG1523 fibroblasts were washed twice with PBS containing 0.1% bovine serum albumin (BSA), incubated for 30 minut~ps on ice with a fluorescein-isothiocyanate (FITC)-labelled anti-human TF monoclonal antibody (4508CJ, American Diagnostica, Greenwich, Ct. USA). The anti-Aspergillus niger glucose oxidase monoclonal IgG1 (Dakopatts) was used as a negative control. Mean channel fluorescence intensity (MFI) and percentage of positive cells were determined for each sample.

Determination of TF activity. The procoagulant activity of TF was determined as described by Lindmark et al.[32]. Briefly, aliquots containing 0.2 x 10^5 AG1518 or AG1523 fibroblasts were washed twice with PBS, placed in the wells of a 96-well microtitreplate (Nunc, Roskilde, Denmark). The procoagulant activity was measured in a two-stage amidolytic assay where a chromogenic substrate, S-2222 (Chromogenix, Mölndal, Sweden), is cleaved by FXa, which in turn is activated from FX by the TF/FVIIa complex. A reaction mixture containing final concentrations of 0.6 mM S-2222, 2 mM $CaCl_2$ and coagulation factors from the factor concentrate Prothromplex-T™ TIM4 (Baxter, Vienna, Austria) at a final concentration of 1 U/ml FVII and 1.2 U/ml FX, was added to the wells, and change in absorbance at 405 nm fol-

lowing a 30 minutes incubation at 37°C was determined. The measurements were done in triplicate.

Chemotaxis assay. The migration response of fibroblasts was assayed by means of the leading front technique in a modified Boyden chamber, as previously described[14,33]. Micropore filters (pore size 8 μm) were coated with a solution of type-1 collagen at room temperature over night. The filters were air dried for 30 minutes immediately before use. Human foreskin fibroblasts AG1523, were grown to confluence in Eagle's MEM supplemented with 10% FBS. The cells were detached by trypsinization (2.5 mg/ml for 10 minutes at 37°C) and suspended in Eagle's MEM with 10% FBS. The fibroblasts were incubated for 10 minutes with or without FVIIa or FFR-FVIIa before assay. One hundred microliters of the cell suspension (2x 10^5 cells/ml) was added above the filter of the Boyden chamber. PDGF-BB was diluted in assay media (Eagle's MEM with 10% FBS) and added below the filter in the chamber. The cells were incubated for 6 hours at 37°C in a humidified chamber containing 95% air/5% CO_2. FVIIa or FFR-FVIIa were present during the entire experiment. The filters were then removed, fixed in ethanol, stained with Mayer's Hemalun, and mounted. Migration was measured as the distance of the two furthest migrating fibroblast nuclei of one high-power field (12.5x 24) in focus. The migration distance in each filter was calculated as the mean of the readings of at least three different parts of the filter. Experiments were performed with two to four separate filters for each concentration of chemoattractant. For each set of experiments, the migration of fibroblasts toward the assay media served as control.
In cases when anti-TF monoclonal antibodies or inhibitors to coagulation factors, TAP and Hirudin, were used, cells were preincubated for 10 minutes with these agents, then with or without FVIIa or FFR-FVIIa before the chemotaxis assay was performed. Antibodies, TAP or Hirudin were also present during the entire chemotaxis experiment. In experiments where the effects on the migration response of different inhibitors, LY294002, U73122 or U73343, were tested, cells were preincubated for 30 minutes with the inhibitors at indicated concentrations, and the inhibitors were also present throughout the experiments.

Assay for release of inositol trisphosphate (IP_3). Six-well plates with semi-confluent cultures of AG1518 human fibroblasts, were incubated over night (approx. 20 hours) with 2 μCi of myo(^{3}H) inositol (Amersham) in 2 ml Ham's F12 with 0.1% FBS. Medium was changed to Ham's F12 with 0.1% FBS (containing 2 mM $CaCl_2$) and 20 mM LiCl and the cells were incubated for 15 minutes at 37°C. Cells were then incubated in the absence or presence of 100

nM FVIIa or 100 nM FFR-FVIIa for one hour. PDGF-BB (0, 10 or 100 ng/ml) was added and the incubation was continued for 10 minutes at 37°C. The IP_3 assay was performed as previously described by Eriksson et al.[34].

Assay for agonist -induced PLC-γ1 phosphorylation. Semi-confluent cultures of AG1518 were serum starved overnight (approx. 20 hours) in medium containing 0.1% FBS, and then incubated in the absence or presence of 100 nM FVIIa or FFR-FVIIa for one hour followed by incubation with 0, 2, 10 or 100 ng/ml PDGF-BB for 5 minutes at 37°C. Cells were lysed and PLC-γ1 was precipitated, essentially as previously described[16], with anti-PLC-γ1 antiserum generated by immunizing rabbits with a peptide corresponding to the carboxyterminus of bovine PLC-γ1[35]. Samples were separated by SDS-PAGE and immunoblotted with the phophotyrosine antibody PY99.

Statistical analysis. Data were analysed using the Statistica TM for Windows package (StatSoft, Tulsa, Okla. USA). A Student's t-test for dependent samples was used to determine statistical significance between different data sets. P values of <0.05 were considered statistically significant.

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References

1. Camerer, E., Kolstø, A-B., Prydz, H. Cell biology of tissue factor, the principal initiator of blood coagulation. *Thromb. Res.* 81, 1-41 (1996).

2. Bazan, JF. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA* 87, 6934-6938 (1990).

3. Rapaport, S.I., Rao, L.V.M. The tissue factor pathway: how it has become a "Prima Ballerina". *Thromb. Haemost.* 74, 7-17 (1995).

4. Kjalke, M., Monroe, D.M., Hoffman, M., Oliver, J.A., Ezban, M., Roberts, H.R. Active site-inactivated factors VIIa, Xa, and IXa inhibit individual steps in a cell-based model of tissue factor-initiated coagulation. *Thromb. Haemost.* 80, 578-584 (1998).

5. Østerud, B. Tissue factor: a complex biological role. *Thromb. Haemost.* 78, 755-758 (1997).

6. Semeraro, N., Colucci, M. Tissue factor in health and disease. *Thromb. Haemost. 78, 759*-764 (1997).

7. Røttingen, J-A., Endens, T., Camerers, E., Iversen, J-G., Prydz, H. Binding of human factor VIIa to tissue factor induces cytosolic Ca^{2+} signals in J82 cells, transfected COS-1 cells, Madin-Darby canine kidney cells and in human endothelial cells induced to synthesize tissue factor. *J. Biol. Chem.* 270, 4650-4660 (1995).

8. Camerer, E., Røttingen, J-A., Iversen, J-G., Prydz, H. Coagulation factors VII and X induce Ca^{2+} oscillations in Madin-Darby canine kidney cells only when proteolytically active. *J. Biol. Chem.* 271, 29034-29042 (1996).

9. Poulsen, L.K., Jacobsen, N., Sørensen, B.B., Bergenhem, N.C.H., Kelly, J.D., Foster, D.C., Thastrup, 0., Ezban, M., Petersen, L.C. Signal transduction via the mitogenactivated protein kinase pathway induced by binding of coagulation factor VIIa to tissue factor. *J. Biol. Chem.* 273, 6228-6232 (1998).

10. Bromberg, M.E., Konigsberg, W.H., Madison, J.F., Pawashe, A., Garen, A. Tissue factor promotes melanoma metastasis by a pathway independent of blood coagulation *Proc. Natl. Acad. Sci. USA* 92, 8205-8209 (1995).

11. Ott, I., Fischer, E.G., Miyagi, Y., Mueller, B.M., Ruf, W. A role for tissue factor in cell adhesion and migration mediated by interaction with actin-binding protein 280. *J. Cell. Biol.* 140, 1241-1253 (1998).

12. Heldin, C-H., Westermark, B. Mechanism of action and in vivo role of platelet-derived growth factor. *Phys. Rev.*, in press 1999.

13. Heldin, C-H., Östman, A., Rönnstrand, L. Signal transduction via platelet-derived growth factor receptors. *Biochim. Biophys. Acta* 1378, 79-113 (1998).

14. Siegbahn, A., Hanimacher, A., Westermark, B., Heldin, C-H. Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. *J. Clin. Invest. 85,* 916-920 (1990).

15. Wennström, S., Siegbahn, A., Yokote, K., Arvidsson, A-K., Heldin, C-H., Mori, S., Claesson-Welsh, L. Membrane ruffling and chemotaxis transduced by the PDGF β-receptor require the binding site for phosphatidylinositol 3'kinase. *Oncogene* 9, 651-660 (1994).

16. Hansen, K., Johnell, M., Siegbahn, A., Rorsman, C., Engström, U., Wernstedt, C., Heldin, C-H., Rönnstrand, L. Mutation of a Src phosphorylation site in the PDGF β-receptor leads to increased PDGF-stimulated chemotaxis but decreased mitogenesis. *EMBO J.* 15, 5299-5313 (1996).

17. Hartzell, S., Ryder, K., Lanahan, A., Lau, L.F., Nathans, D. A growth factor-responsive gene of murine Balb/c 3T3 cells encodes a protein homologous to human tissue factor. *Mol. Cell. Ciol.* 9, 2567-2573 (1989).

18. Luther, T., Flössel, C., Mackman, N., Bierhaus, A., Kasper, M., Albrecht, S., Sage, H.E~., Iruela-Arispe, L., Grossmann, H., Ströhlein, A., Zhang, Y., Nawroth, P.P., Carmeliet, P., Loskutoff, D.J., Müller, M. Tissue factor expression during human and mouse development. *Am. J. Pathol.* 149, 101-113 (1996).

19. Bugge, T.H., Xiao, Q., Kombrinck, K.W., Flick, M.J., Holmbäck, K., Danton, M.J.S., Colbert, M.C., Witte, D.P., Fujikawa, K., Davie, E.W., Degen, J.L. Fatal embryonic bleeding events in mice lacking tissue factor, the cell-associated initiator of blood coagulation. *Proc. Natl. Acad. Sci. USA* 93, 6258-6263 (1996).

20. Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., van Vlaenderen, I., Demunck, H., Kasper, M., Breier, G., Evrard, P., *et al.* Role of tissue factor in embryonic

blood vessel development *Nature* 383, 73-75 (1996).

21. Zhang, Y., Deng, Y., Luther, T., Muller, M., Ziegler, R., Waldherr, R., Stern, D.M., Nawroth, P.P. Tissue factor controls the balance of angiogenic and antiangiogenic properties of tumor cells in mice. *J. Clin. Invest.* 94, 1320-1327 (1994).

22. Mueller, B.M., Ruf, W. Requirement for binding of catalytically active factor VIIa in tissue factor-dependent experimental metastasis. *J. Clin. Invest. 101*, 1372-1378 (1998).

23. Rönnstrand, L., Siegbahn, A., Rorsman, C., Johnell, M., Hansen, K., Heldin, C-H. Overactivation of phospholipase C-γl renders PDGF β-receptor expressing cells independent of the PI3'-kinase pathway for chemotaxis. *J. Biol. Chem.*, in press 1999.

24. Müller, M., Albrecht, S., Gölfert, F., Hofer, A., Funk, R.H.W., Magdolen, V., Flössel, C., Luther, T. Localisation of tissue factor in actin-filament-rich membrane areas of epithelial *cells. Exp. Cell. Res,* 248, 136-147 (1999).

25. Lauffenburger, D.A., Horwitz, A.F. Cell migration: a physically integrated molecular process. *Cell* 84, 359-369 (1996).

26. Higaki, M., Sakaue, H., Ogawa, W., Kasuga, M., Shimokado, K. Phosphatidylinositol 3-kinase-independent signal transduction pathway for platelet-derived growth factor induced chemotaxis. *J. Biol. Chem.* 271, 29342-29346 (1996).

27. Kundra, V., Escobedo, J.A., Kazlauskas, A., Kun Klm, H., Goo Rhee, S., Williams, L.T., Zetter, B.R. Regulation of chemotaxis by the platelet-derived growth factor receptor-β. *Nature* 367, 474-476 (1994)

28. Jang, Y., Guzman, L.A., Lincoff, A.M., Gottsauner-Wolf, M., Forudi, F., Hart, C.E., Courtman, D.W., Ezban, M., Ellis, S.G., Topol, E.J. Influence of blockade at specific levels of the coagulation cascade on restenosis in a rabbit atherosclerotic femoral artery injury model. *Circulation* 92, 3041-3050 (1995).

29. Thim, L., Christensen, M., Nicolaisen, E.M., Lund-Hansen, T., Pedersen, A.H., Hedner, U. Amino acid sequence and posttranslational modification of human factor VII from plasma and transfected baby hamst r kidney cells. *Biochemistry* 27, 7785-7793 (1988).

30. Sorensen, B.B., Persson, E., Freskgård, P.O., Kjalke, M., Ezban, M., Williams, T., Rao, L.V.M. Incorporation of an active site inhibitor in factor FVIIa alters the affinity for tissue factor. *J. Biol. Chem.* 272, 11863-11868 (1997).

31. Morrissey, J.H., Fair, D.S., Edgington, T.S. Monoclonal antibody analysis of purified and cell-associated tissue factor. *Thromb. Res.* 52, 247-261 (1988).

32. Lindmark, E., Tenno, T., Chen, J., Siegbahn, A. IL-10 inhibits LPS-induced human monocyte tissue factor expression in whole blood. *Br. J. Haematol.* 102, 597-604 (1998).

33. Nistér, M., Hammacher, A., Mellström, K., Siegbahn, A., Rönnstrand, L., Westermark, B., Heldin, C-H. A glioma-derived PDGF A chain homodimer has different functional activities from a PDGF AB heterodimer purified from human platelets. *Cell* 52, 791-799 (1988).

34. Eriksson, A., Nånberg, E., Rönnstrand, L., Engström, U., Hellman, U., Rupp, E., Carpenter, G., Heldin, C-H., Claesson-Welsh, L. Demonstration of functionally different interactions between phospholipase C-γ and the two types of platelet-derived growth factor receptors. *J. Biol. Chem.* 270, 7773-7781 (1995).

35. Artega, C.L., Johnson, M.D., Todderud, G., Coffey, R.J., Carpenter, G., Page, D.L. Elevated content of the tyrosine kinase substrate phospholipase C-γl in primary human breast carcinomas. *Proc. Natl. Acad. Sci. USA* 88, 10435-10439 (1991).

CLAIMS

1. A method for regulating cell migration, comprising the steps of:
a) contacting said cell with factor VIIa or a tissue factor antagonist;
b) determining the migration of said cell.

2. The method of claim 1, wherein said cell is a human cell expressing tissue factor, including fibroblasts, smooth muscle cells, tumour cells, haematopoietic cells and epithelial cells.

3. The method of claim 2, wherein the tissue factor antagonist is modified factor VIIa known as factor VIIai.

4. The method according to claim 3, wherein the modified factor VII is selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethylketone.

5. A method of enhancing cell migration, comprising contacting the cell with FVIIa or a tissue factor agonist.

6. A method of reducing or inhibiting cell migration, comprising contacting the cell with a tissue factor antagonist.

7. A method for inducing or enhancing wound healing in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition comprising Factor VIIa or a tissue factor agonist.

8. A method for inhibiting the invasiveness of tumour cells comprising contacting said cells with an effective amount of a tissue factor antagonist.

9. A method for inhibiting cell migration, invasion, migration-induced cell proliferation or angiogenesis in a subject having a disease or condition associated with undesired cell migration, invasion, migration-induced cell proliferation or angiogenesis, comprising

administering to said subject an effective amount of a pharmaceutical composition comprising a tissue factor antagonist.

10. A method according to claim 9, wherein the disease or condition is primary tumour growth, tumour invasion or metastasis.

11. A method according to claim 10, wherein the tissue factor antagonist is modified factor VII known as FVIIai.

12. Use of factor VIIa or a tissue factor antagonist for the manufacture of a medicament for regulating cell migration.

13. Use according to claim 12, wherein factor VIIa is used for the manufacture of a medicament for enhancing cell migration.

14. Use according to claim 12, wherein a tissue factor antagonist is used for the manufacture of a medicament for reducing or inhibiting cell migration.

15. The method of claim 14, wherein the tissue factor antagonist is modified factor VIIa known as factor VIIai.

16. Use according to claim 15, wherein the modified factor VII is selected from Dansyl-Phe-Pro-Arg chloromethyl ketone, Dansyl-Glu-Gly-Arg chloromethyl ketone, Dansyl-Phe-Phe-Arg chloromethyl ketone and Phe-Phe-Arg chloromethylketone.

ABSTRACT

The present invention relates to usage of FVII and/or FVIIa and/or another TF agonist and/or FVIIai and/or another TF antagonist in therapeutic treatment of pathological conditions that can be related to cell migration or treated by specific regulation of cell migration or chemotaxis.

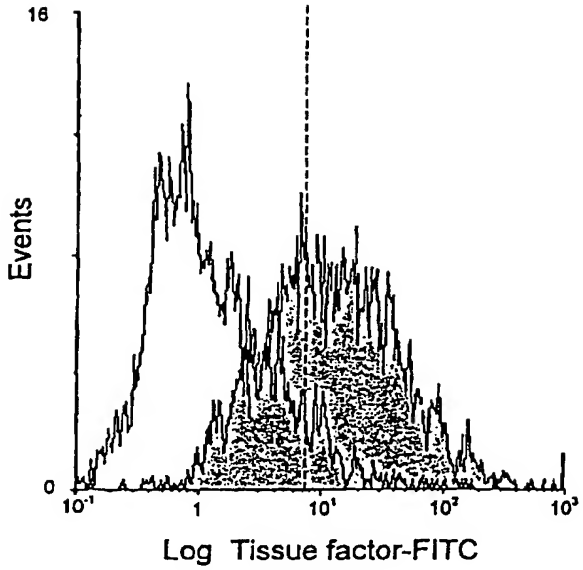

16
Events
0
10^{-1}
1
10^{1}
10^{2}
10^{3}
Log Tissue factor-FITC

Fig. 1A

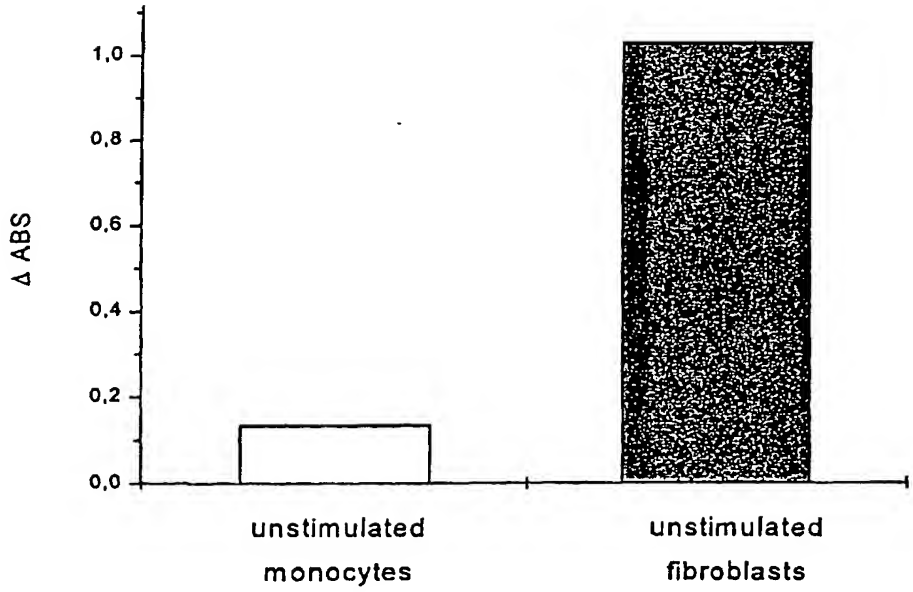
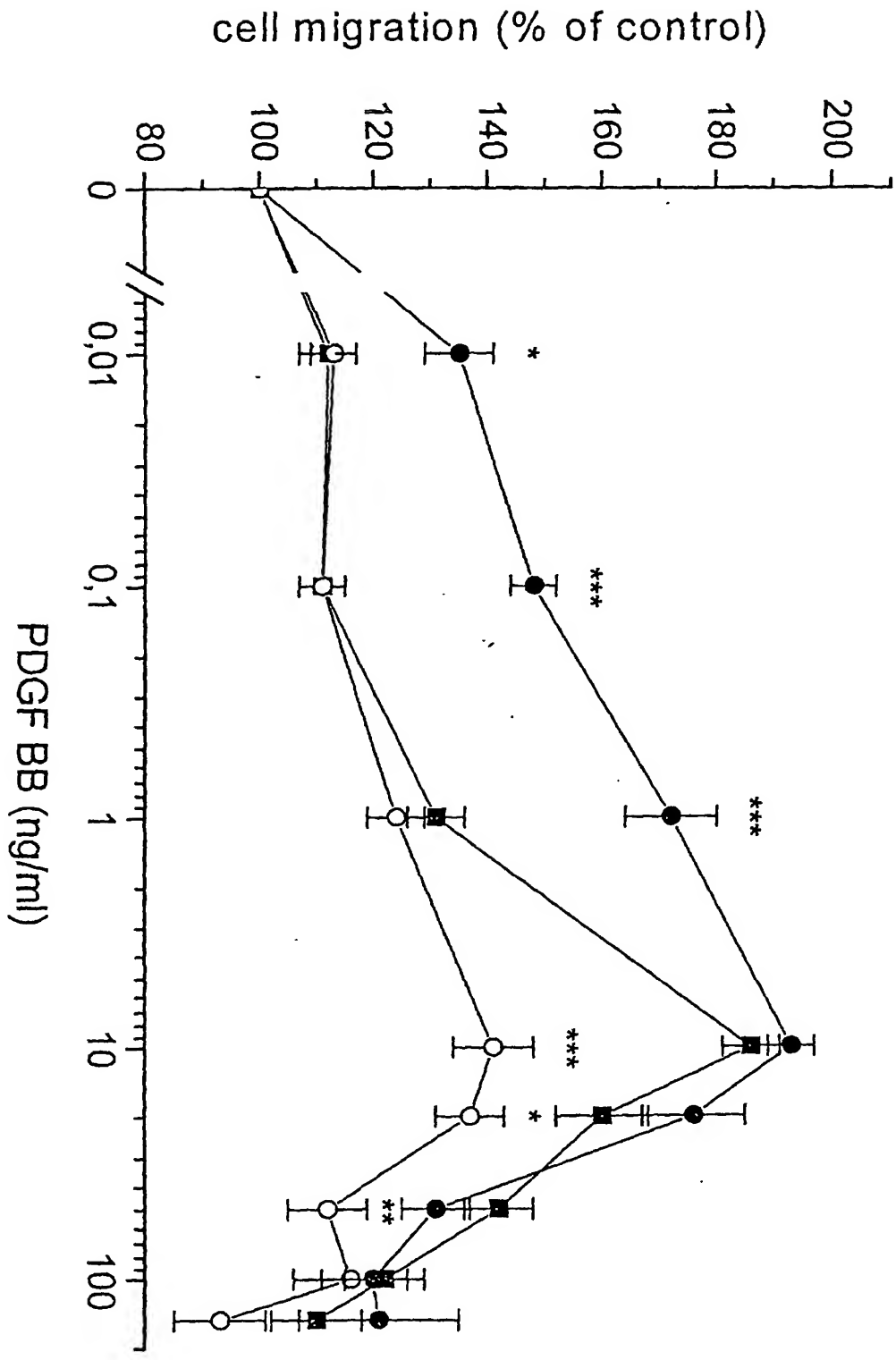

Δ ABS
1,0
0,8
0,6
0,4
0,2
0,0
unstimulated monocytes
unstimulated fibroblasts

Fig. 1B


cell migration (% of control)
80
100
120
140
160
180
200
0
0,01
0,1
1
10
100
PDGF BB (ng/ml)
*
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***
***
*
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Fig. 2

Fig. 3A

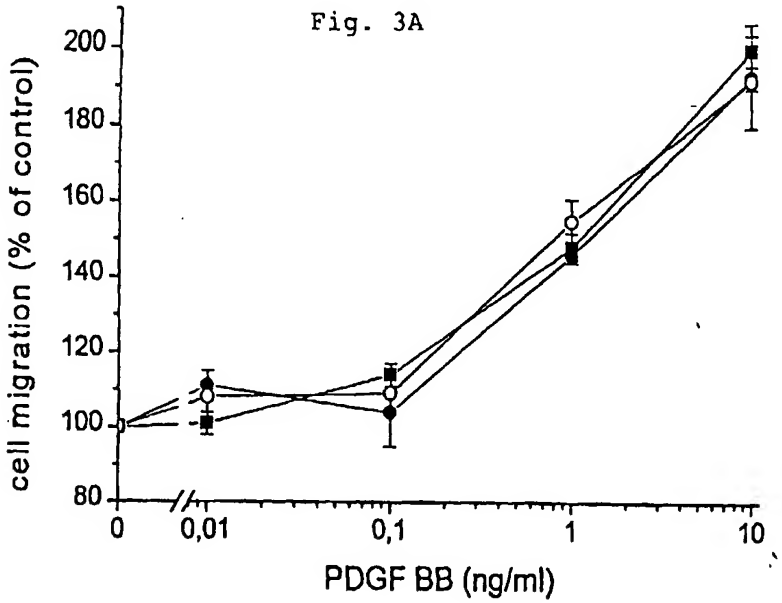


Fig. 3C

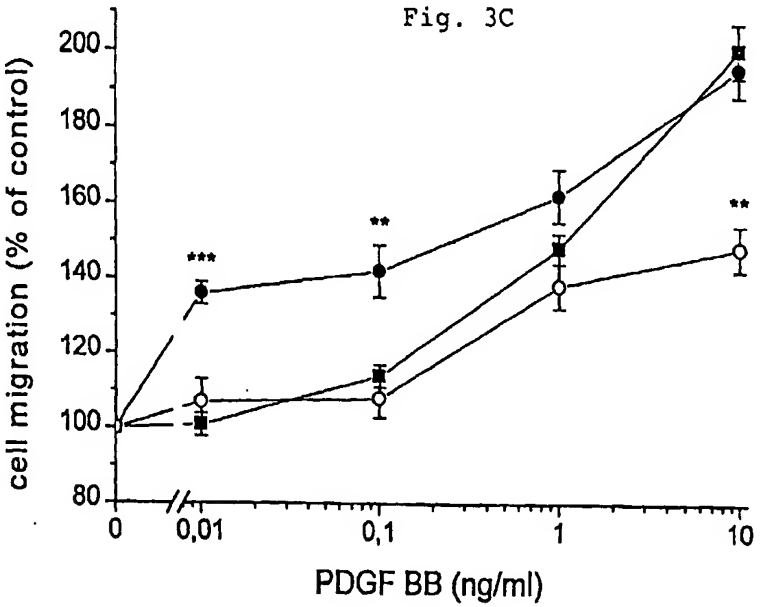


Fig. 3B

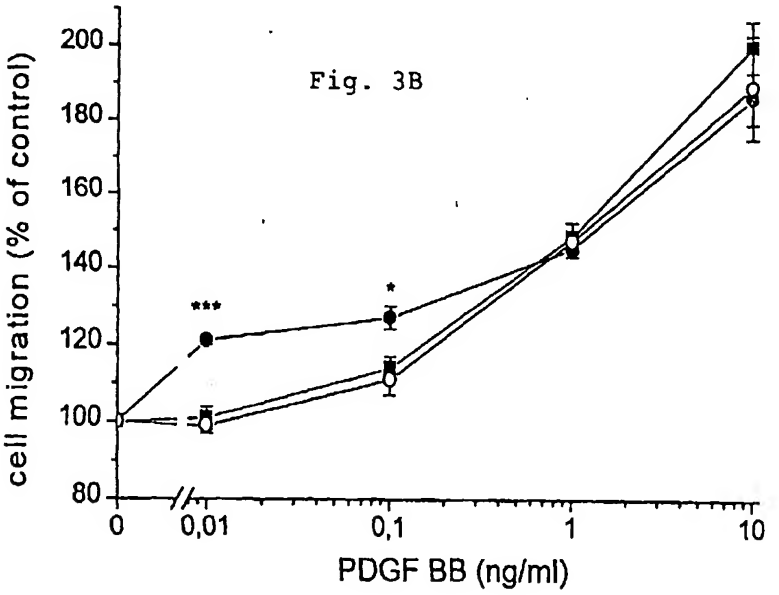
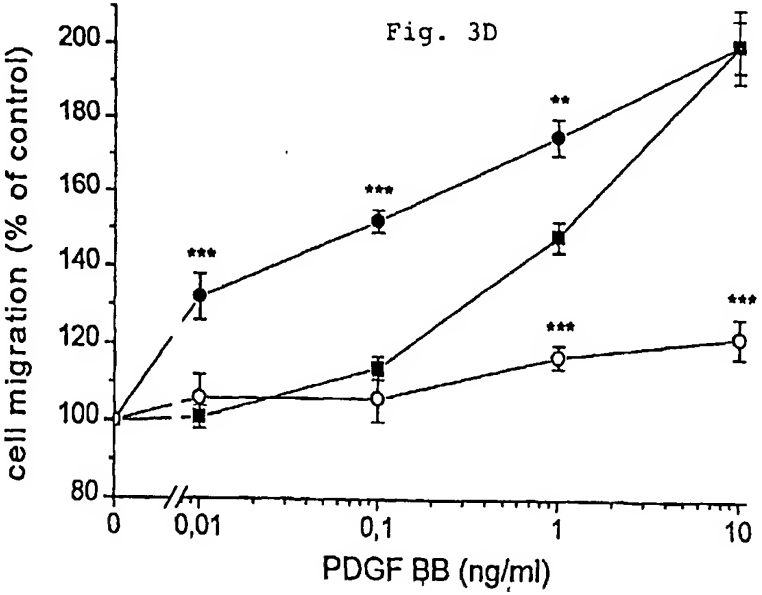


Fig. 3D



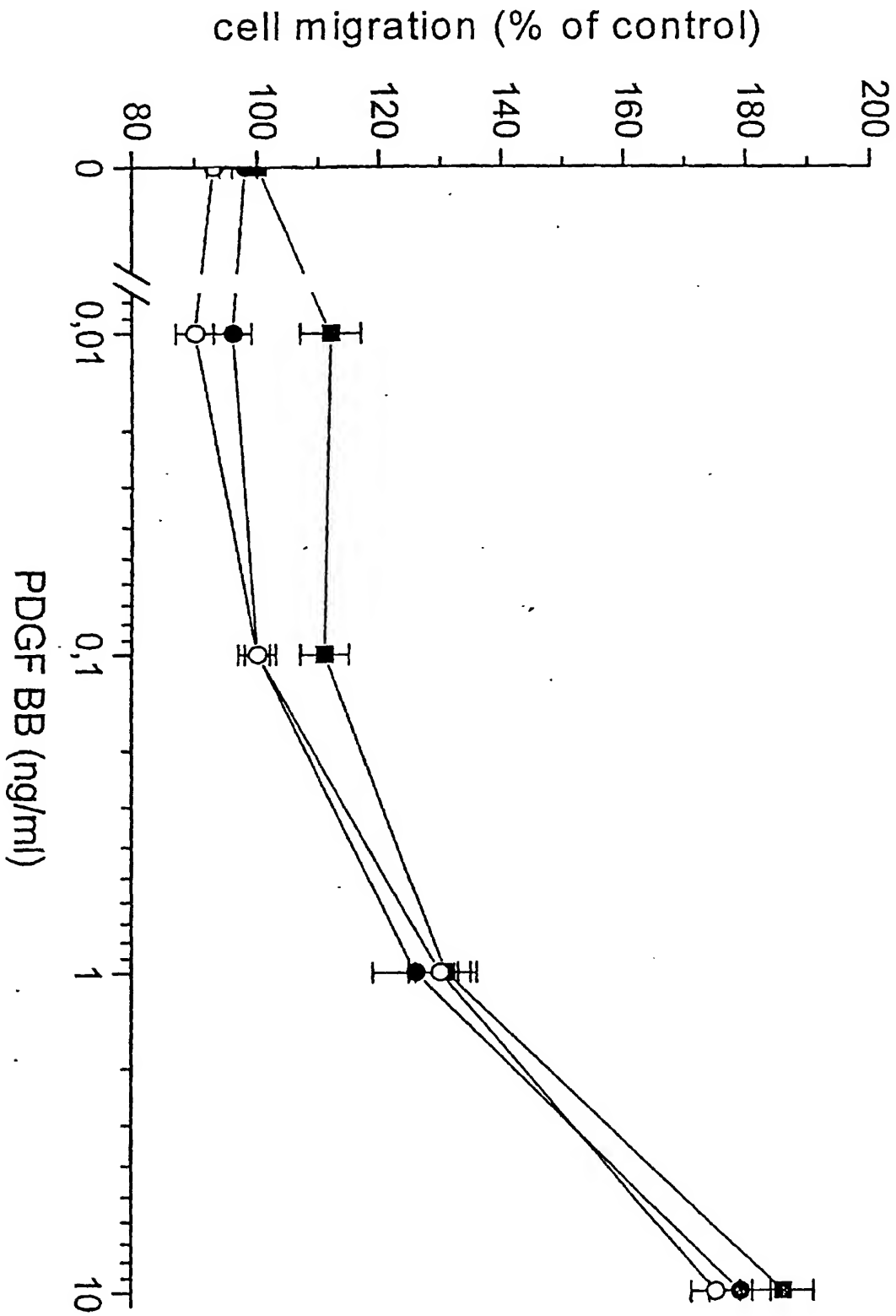

cell migration (% of control)
80
100
120
140
160
180
200
0
0,01
0,1
1
10
PDGF BB (ng/ml)

Fig. 4

Fig. 5A

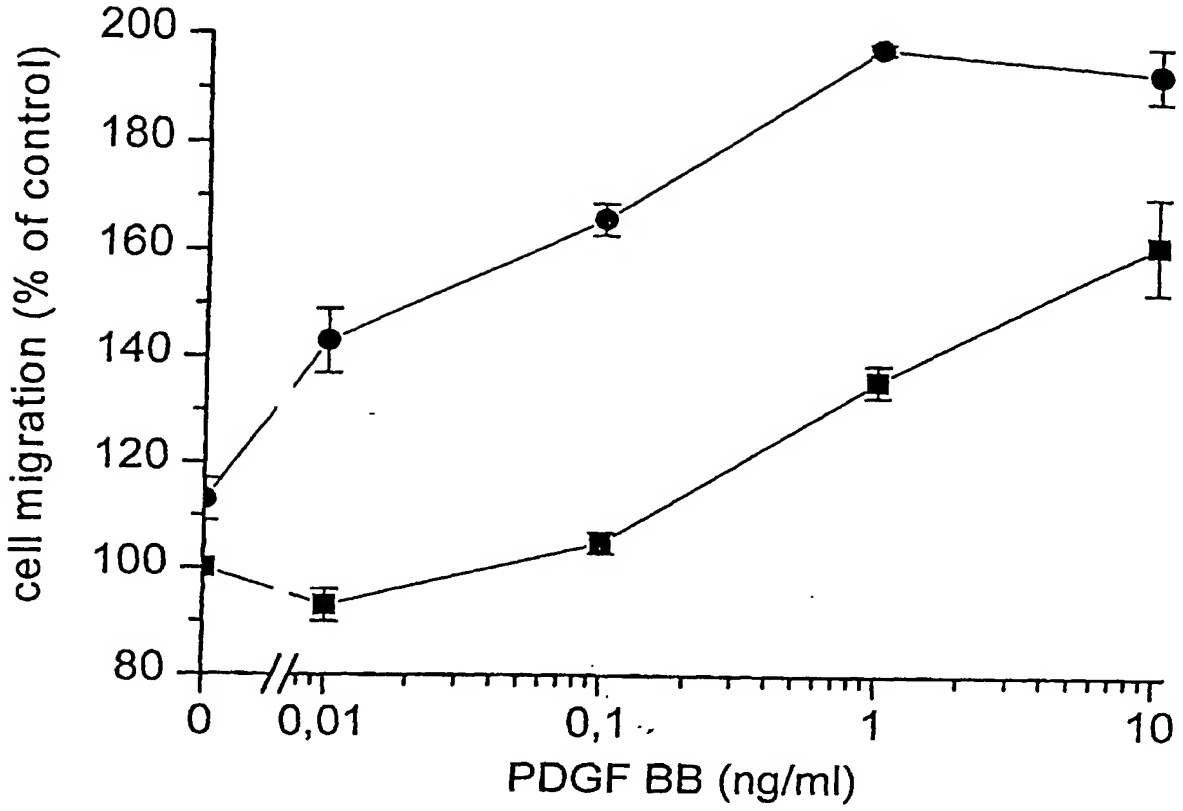
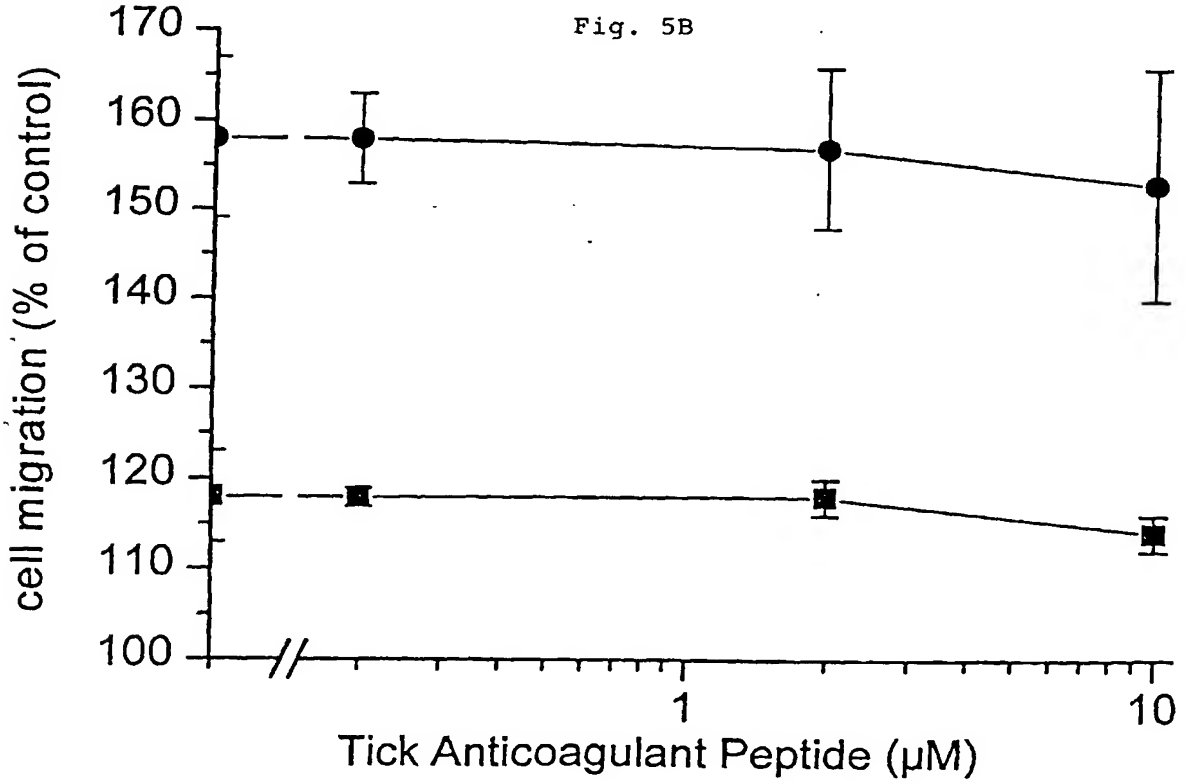

200
180
160
140
120
100
80
cell migration (% of control)
0
0,01
0,1
1
10
PDGF BB (ng/ml)

Fig. 5B


170
160
150
140
130
120
110
100
cell migration (% of control)
1
10
Tick Anticoagulant Peptide (μM)

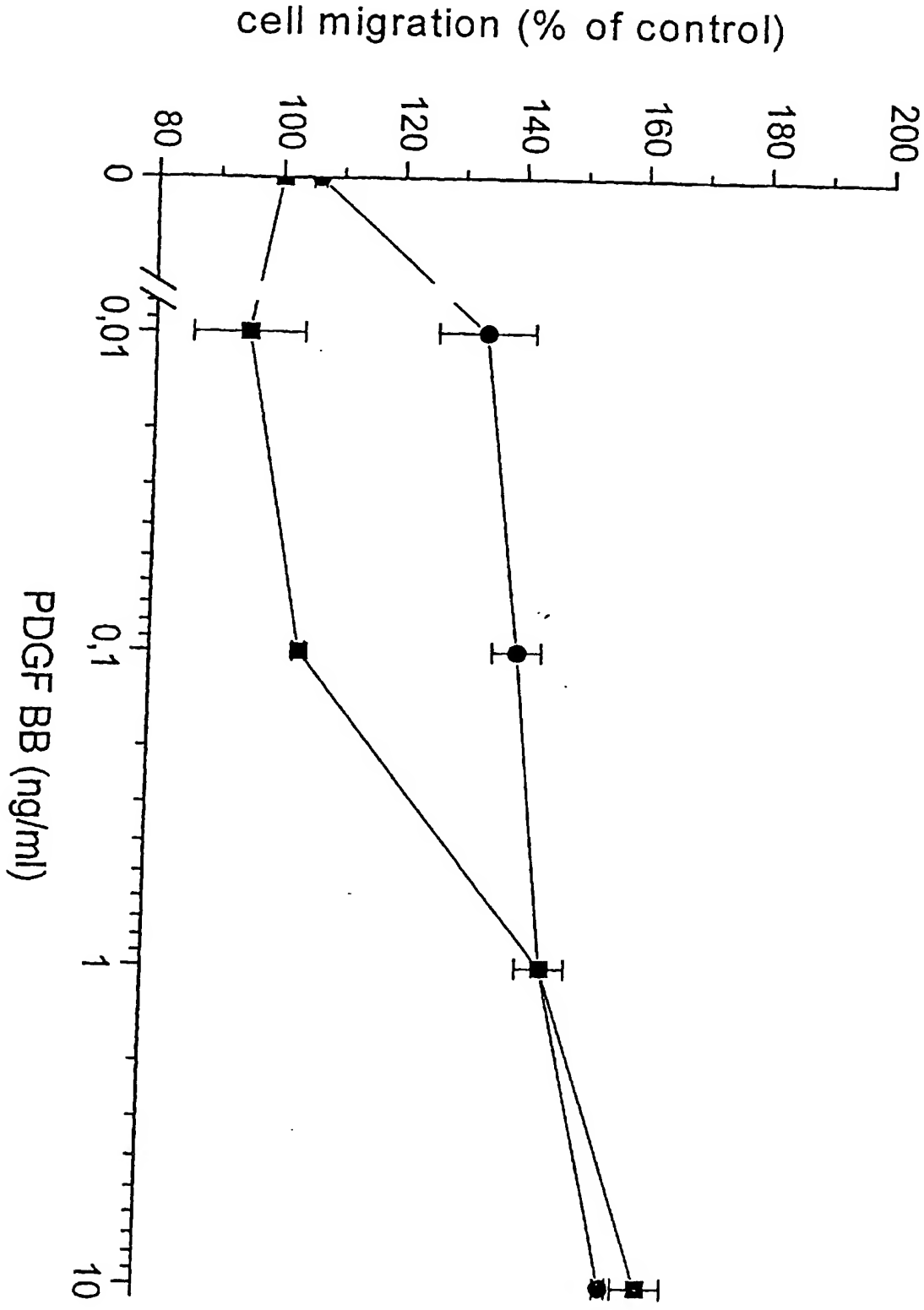

cell migration (% of control)
80
100
120
140
160
180
200
0
0,01
0,1
1
10
PDGF BB (ng/ml)

Fig. 6

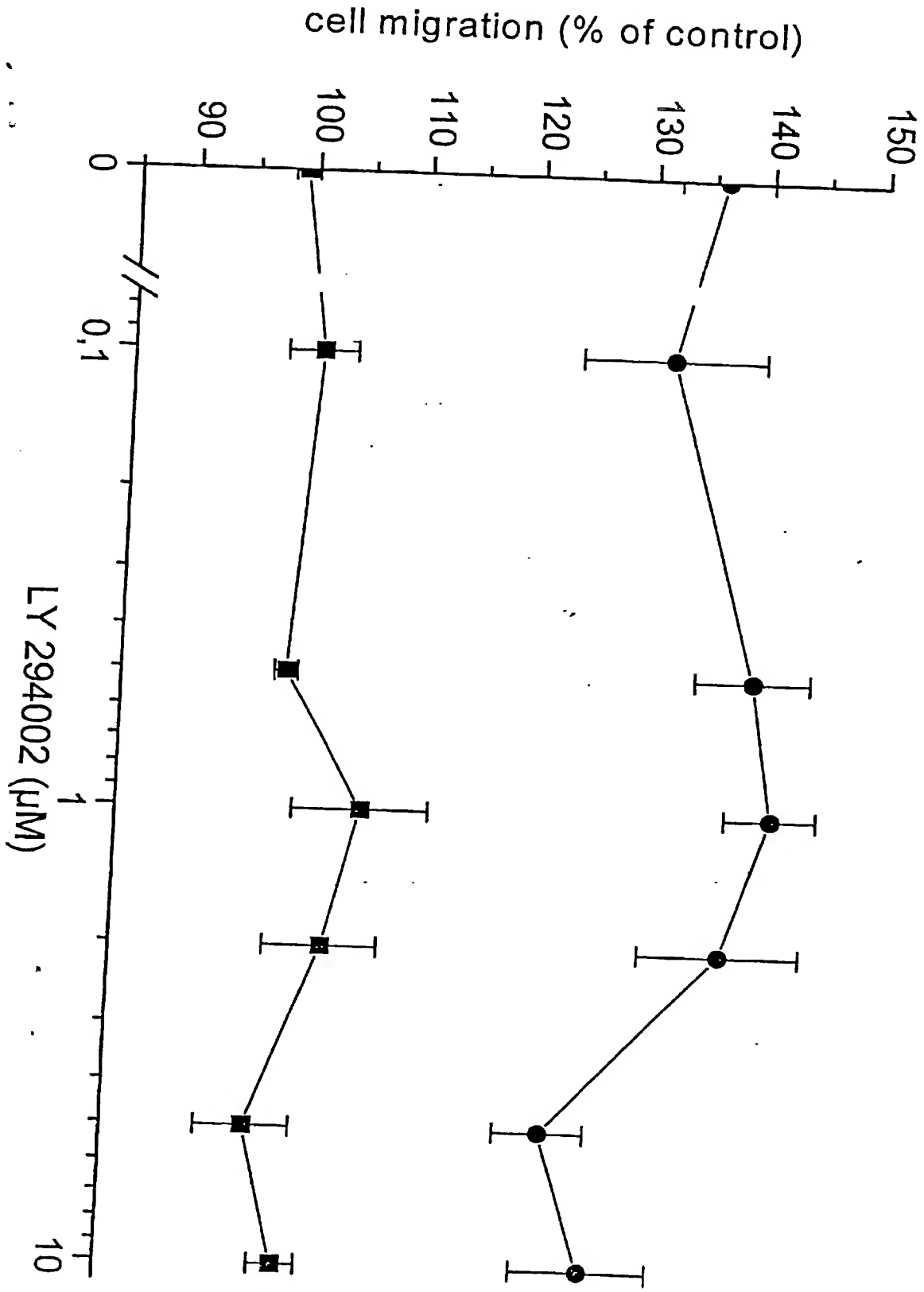

cell migration (% of control)
90
100
110
120
130
140
150
0
0,1
1
10
LY 294002 (µM)

Fig. 7